Effect of Mineral Dusts on Metabolic Cooperation between Chinese Hamster V79 Cells *in Vitro*

by M. Chamberlain*

The mode of action of inorganic fibers as carcinogens is unknown. There is conflicting evidence whether they act by inducing lesions in DNA, such as initiating carcinogens, or alternatively act as promoters of carcinogenesis. Recent evidence has suggested that promoters inhibit the process of cell-to-cell chemical communication known as metabolic cooperation.

Chinese hamster V79-4 cells in culture exhibit metabolic cooperation which can be blocked by the promoter tetraphorbol-13-acetate (TPA). Three kinds of mineral dust were tested to determine whether or not they could interfere with the intercellular communication. The three dusts studied were: amosite, a fibrous asbestos from the Union Internationale Contre le Cancer (UICC) standard reference series, which is a typical carcinogenic fibrous dust inducing pleural tumors following intrapleural injection into rats; ball-milled amosite, a nonfibrous dust derived from UICC amosite by ball-milling, which has the physical properties characteristic of a noncarcinogenic dust; and Min-U-Sil silica, a widely available sample of fibrogenic quartz which does not induce pleural tumors following intrapleural injection into rats. The results demonstrated that metabolic cooperation between V79-4 cells cannot be reduced by carcinogenic asbestos fibers or by relatively large numbers of nontoxic dust particles. It is concluded that if inorganic fibers promote carcinogenesis, then they do not operate by blocking metabolic cooperation.

Introduction

Inorganic fibers are carcinogenic in man and experimental animals. Exposure of man to airborne asbestos can lead to development of bronchogenic carcinoma and pleural and peritoneal mesothelioma (1,2). In experimental animals, the sizes of durable inorganic fibers have been shown to determine their carcinogenic potentials. The probability of a dust inducing mesothelioma following intrapleural implantation in experimental animals depends upon the number of fibers longer than about 8 μm and thinner than about 1.5 μm per unit mass. This relationship between fiber dimensions and carcinogenic potential has been demonstrated for fibers of different compositions, such as borosilicate glass, silicon carbide, potassium octatitanate and dihydroxy sodium aluminum carbonate (3-5). *In vitro* experiments have demonstrated that fibers of sizes similar to those known to be carcinogenic in experimental ani-

mals are toxic towards established cell lines, mouse peritoneal macrophages and a macrophagelike cell line (6-10). The toxicity of fibrous dusts of different compositions is determined largely by fiber size but other factors, such as culture conditions, can exert significant effects on the relative toxicities of different dusts (11).

This mode of action of carcinogenic inorganic fibers is not known. Several investigations have been conducted to determine whether or not inorganic fibers act as initiating agents by inducing lesions in DNA. Asbestos and glass fibers are inert in bacterial mutation tests (12,13), but there are reports that asbestos induces mutations at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster cells (14,15). Asbestos does not induce DNA damage as detected by induction of unscheduled DNA synthesis (16). Chromosome aberrations are induced in mammalian cells by carcinogenic asbestos and glass fibers (7,14,17,18), whereas noncarcinogenic fibers and isometric silica particles do not induce such aberrations. There is conflicting evidence whether or not inorganic fibers induce sister-chromatid exchanges (19-22).

*MRC Pneumoconiosis Unit, Llandough Hospital, Penarth, South Glamorgan, CFG IXW, U.K. Present address: Environmental Safety Laboratory, Unilever Research, Colworth House, Sharnbrook, Bedford MK44 1LQ, U.K.

From the present evidence it is not clear whether inorganic fibers exert their carcinogenic effects by inducing lesions in DNA or alternatively act more like tumor promoters. Studies of the mode of action of promoters have demonstrated that they exert several biological effects including stimulation of ornithine decarboxylase, induction of plasminogen activator, inhibition of cell differentiation, induction of sister-chromatid exchanges and increasing transformation frequencies of mutagens *in vitro* (23). It is not clear which of these activities is implicit in tumor promotion.

Several tumor promoters have recently been reported to inhibit the process of cell-to-cell chemical communication, known as metabolic cooperation mediated through cell contact (24-26). This study was undertaken to determine whether or not a typical carcinogenic fibrous dust, namely UICC amosite, could inhibit metabolic cooperation in V79-4 cultures. Two dusts which possess physical characteristics typical of non-carcinogenic dusts were included to control for the possibility that dust particles may physically interfere with the establishment of metabolic cooperation.

Materials and Methods
Cells and Media

V79-4 Chinese hamster lung cells (27) were obtained from Dr. C. Arlett (MRC Cell Mutation Unit, Brighton, U.K.) and were grown in Eagles Minimal Essential Medium (MEM) supplemented with 15% fetal calf serum, 2 mM L-glutamine and antibiotics. Cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air. Azaguanine-resistant cells (AGr) were derived from V79-4 cells by cloning using 30 µg/mL 8-azaguanine as the selective agent. The clone of cells was disaggregated by using trypsin-EDTA and a large stock of cells grown in medium containing the selective agent. Several ampules of this stock of cells were frozen in liquid nitrogen. AGr cells exhibited the same plating efficiency (about 80%) in medium containing 8-azaguanine as V79-4 cells in medium without the selective agent.

Test Agents

12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was obtained from P-L Biochemicals Inc. (Milwaukee, WI, USA) and was dissolved and diluted in dimethyl sulfoxide (DMSO) for use in the experiments. The amosite asbestos was from the UICC standard reference series (28). The milled amosite was prepared from UICC amosite by milling in a ceramic ball mill for a total of 4 hr by using the method described previously (6). Min-U-Sil silica was obtained from the Pennsylvania Silica Sand Co. Weighed amounts of each dust were sterilized by autoclaving, and suspensions of 1 mg/mL were prepared in medium immediately before use. The dusts were dispersed by treatment in an ultrasonic waterbath for 1 min. The size characteristics of the dust samples are shown in Table 1.

Cytotoxicity

The survival of V79-4 or AGr cells in the absence and presence of the test agents was determined by cloning efficiency from single cell suspensions. This method has been described previously (29) with the modification that the colonies were counted using an automatic colony counter.

Assay for Metabolic Cooperation

The cloning efficiency of AGr cells in the absence and presence of 8-azaguanine-sensitive V79-4 cells was determined for each treatment. The number of AGr colonies which developed in the presence of V79-4 cells was expressed as a percentage of the number of AGr colonies which developed in the absence of V79-4 cells.

A suspension of 50 AGr cells/mL was prepared in a large volume of medium containing 30 µg/mL 8-azaguanine and 20 mL volumes dispensed into a series of paired universal bottles. An appropriate amount of the test agent was added to each universal and the contents mixed by inversion; controls received solvent or medium only. The influence of each treatment on the survival of AGr Cells in the

Table 1. Size characteristics of dust samples.

| Dust | Number of fibers per µg | | | |
	Total fibers	Fibers > 6.5 µm	Nonfibrous particles	All particles
UICC amosite[a]	3.10×10^5	6.82×10^4	2.25×10^5	5.35×10^5
Ball-milled amosite (4 hr)[a]	1.28×10^5	$<< 1.28 \times 10^1$	2.73×10^7	2.74×10^7
Min-U-Sil silica[b]	0	0	5.90×10^5	5.90×10^5

[a] Unpublished data.
[b] Data from Wagner et al. (33).

Table 2. Recovery of AGr cells after various treatments.

Treatment	Treatment level, µg/mL	Recovery of AGr cells (± 95% confidence limits), %
Control (+ DMSO)		44.9 ± 6.6
TPA	0.01	91.0 ± 7.8
Control		38.4 ± 6.1
UICC amosite	0.01	42.9 ± 5.3
	0.1	33.9 ± 5.2
	1.0	29.4 ± 5.0
	5.0	38.6 ± 4.3
Milled amosite	0.01	52.6 ± 8.9
	0.1	49.4 ± 4.9
	1.0	51.0 ± 8.9
	5.0	40.8 ± 5.0
Min-U-Sil	5.0	53.8 ± 11.6
	10.0	52.9 ± 9.7
	25.0	36.5 ± 5.5
	50.0	24.9 ± 9.9

absence of V79-4 cells was determined by plating the contents of one of each pair of universal bottles into four 6-cm diameter Petri dishes. The remaining universals received 2×10^6 V79-4 cells before the contents were similarly dispensed. After incubation for 6 days, the colonies were fixed, stained and counted.

Measurement of Projected Surface Area of AGr Cells

10^5 AGr cells were allowed to attach to and spread onto the surface of a 9-cm diameter Petri dish for 5 hr, and the cells were then fixed and stained. The diameters of 100 attached cells were measured at $\times 400$ magnification by using an eyepiece graticule which had been calibrated with the use of a stage micrometer.

Results

Effects of Agents on Metabolic Cooperation

The influence of the test agents on metabolic cooperation in V79-4 cultures is shown in Table 2. Establishment of metabolic cooperation resulted in recovery of less than half of the AGr colonies, whereas TPA, which was used as a positive control, inhibited the cooperation and led to the recovery of virtually all (91.0%) of the AGr colonies. At 0.01 µg/mL, TPA was not toxic to the cells.

The three dusts tested (UICC amosite, ball-milled amosite and Min-U-Sil) did not allow significantly greater recovery of AGr colonies compared with their controls (Newman-Keuls test, $p > 0.05$) and so did not inhibit metabolic cooperation. Particles of all three types of dusts were found to be associated with the cells. Milled amosite and Min-U-Sil had no discernible effects on the morphology of the cells.

Table 3. Number of dust particles/cell.

Dust		Number of stated particle type per cell	
Type	Concn, µg/mL	All particles	Fibers>6.5µm
UICC amosite	5	2.15	0.27
Milled amosite	5	108.6	< 0.001
Min-U-Sil	50	23.6	0

However, at the higher concentrations of UICC amosite, occasional "giant cells" were observed in the AGr colonies at the end of the experiment (29).

Estimates of the Number of Dust Particles per Cell

The presence of large numbers of dust particles in V79-4 cultures may have prevented cells from establishing metabolic cooperation merely by their physical presence in the cultures if not through some (postulated) specific membrane interactions. In order to take account of this possibility, estimates of the number of fibers or particles which would settle onto the cells by sedimentation were made (see Table 3). Measurements showed that the projected surface area of an attached AGr cell was about 460 µm². The figures presented in Table 3 can be taken only as estimates of minimum doses, because further interactions between some cells and dust particles can occur on the Petri dish surface.

Discussion

UICC amosite was chosen for this study because it is a typical carcinogenic fibrous dust which has been studied extensively both *in vivo* (30,31) and in cultures of V79-4 cells (6,29). The toxicity of UICC amosite towards V79-4 cells has been ascribed to those fibers longer than about 6.5 µm (6). Ball-milled

amosite was included to control for the effects of mineral particles of similar composition to, but having different physical properties from, UICC amosite. Min-U-Sil was included as a nonfibrous dust which does not induce mesotheliomas following intrapleural injection (*32*) and is relatively inert towards V79-4 cells (*29*).

The compounds which have been reported to reduce metabolic cooperation in cultures of mammalian cells do so at nontoxic concentrations (*24,25*). All three dusts were tested at nontoxic doses but to control for the possibility that UICC amosite was a very weak inhibitor of the communication it was also tested at a toxic dose of 5 μg/mL (70% survival). None of the dusts inhibited metabolic cooperation in the V79-4 cultures (Table 2), whereas TPA effectively blocked the communication at 0.01 μg/mL as has been reported previously (*24*). The data presented in Table 3 illustrate that over 100 particles of milled amosite or 23 particles of Min-U-Sil per cell cannot prevent V79-4 cells from establishing metabolic cooperation.

The results reported here demonstrate that metabolic cooperation between V79-4 cells cannot be reduced by carcinogenic asbestos fibers or by relatively large numbers of nontoxic dust particles, and it is suggested that if inorganic fibers promote carcinogenesis, then they do not operate by blocking metabolic cooperation. This does not preclude inorganic fibers from acting as promoters by other mechanisms such as by affecting metabolism which may lead to the increased yield of initiating molecules.

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